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Different Modes of Cell-killing Action between DNA Topoisomerase I and II Inhibitors Revealed by Kinetic Analysis

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We compared the modes of cell-killing by DNA topoisomerase I and II inhibitors. The effects of camptothecin (CPT), KT-6528 and UCE6 upon colony formation by inhibiting DNA topoisomerase I, and of etoposide (VP-16), teniposide, amsacrine and UCT4-A as inhibitors of DNA topoisomerase II were analyzed based upon a kinetic method that distinguishes between cell cycle phase-specific and -nonspecific agents. Human colorectal cancer WiDr cells were exposed to several concentrations of each agent for various periods and 90%-inhibitory concentrations (IC_{90}) at each time were determined by means of a clonogenic assay. When exposure times and corresponding IC_{90}s were plotted on a log-log scale, all inhibitors of DNA topoisomerase II gave curves including a linear portion with a slope of -1, which is characteristic of cell cycle phase-nonspecific agents. In contrast, the curves for all inhibitors of DNA topoisomerase I had a much steeper slope than -1, which is typical of cell cycle phase-specific agents. In agreement with this finding, the cells were remarkably accumulated in the G_2-M phase when exposed to VP-16, but in late S-phase when exposed to CPT as determined by a flow cytometric assay. These results indicated that the two classes of agents kill cells in a quite different manner although they are inhibitors of similar enzymes.

Key words: DNA topoisomerase inhibitor — Camptothecin — Etoposide — Cell killing action — Kinetic analysis

Two types of DNA topoisomerases are regarded as targets of cancer chemotherapy. CPT[4] inhibits DNA topoisomerase I, and two major analogs of CPT, irinotecan and topotecan, reportedly possess significant clinical therapeutic activities against some solid cancers. On the other hand, the antitumor epipodophyllotoxins, VP-16 and VM-26, and DNA intercalators such as m-AMSA interfere with DNA topoisomerase II.[1-4] In the broad sense, all these drugs are inhibitors of DNA topoisomerase. However, recent biochemical studies have indicated that these inhibitors have different modes of cell-killing depending on the subtype of the target enzyme.[5-9] Thus, it is necessary to differentiate the features of these inhibitors.

We have developed a kinetic analysis of cell-killing which distinguishes between cell cycle phase-specific and -nonspecific agents. We proved that the cell-killing effect of the latter depends on the product of concentration and time or AUC and in contrast, that of the former does not.[10,11] We demonstrated that 5-fluorouracil acts in a different manner depending upon the length of cell exposure to it.[12] The purpose of the present study was to apply our kinetic analysis to two classes of inhibitors of DNA topoisomerase, and to compare the modes of cell-killing.

MATERIALS AND METHODS

Chemicals CPT was purchased from Sigma Chemical Co., St. Louis, MO. KT-6528,[13] UCE6[14] and UCT4-A[15] were prepared as previously described. VP-16, VM-26 and m-AMSA were gifts from Nippon Kayaku Co., Ltd., Tokyo, Bristol-Myers Co., Syracuse, NY, and Dr. M. Yamato, Okayama University of Science, Okayama, respectively.

Cell line The human colon cancer line WiDr was obtained from the American Type Culture Collection (Rockville, MD) and maintained in minimum essential medium supplemented with 10% fetal bovine serum and a 1% antibiotic-antimycotic solution (Sigma Chemical Co.).

Colony-forming assay Cells harvested by trypsinization were seeded at a density of 200, 400 or 800 cells in 35-mm dishes in a total volume of 1.8 ml. On the day after seeding, 0.2 ml of a drug solution was added to each dish, and the cultures were incubated for various periods. Dishes containing higher density of cells were used as the drug concentration was increased. The plates were

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[4] The abbreviations used are: CPT, camptothecin; VP-16, etoposide; VM-26, teniposide; m-AMSA, amsacrine; UCT4-A, terpentecin; BrdUrd, 5-bromo-2′-deoxyuridine; PBS, phosphate-buffered saline; IC_{90}, concentration necessary for 90% cell kill; AUC, the area under the drug concentration-time curve; FCM, flow cytometry.

washed twice with 2 ml of PBS, and then 2 ml of culture medium was added to each. On the 10th or 11th day after seeding, the cells were washed with PBS, fixed with 10% formalin, and stained with 0.05% crystal violet. Colonies were counted using a Colony Analyzer CA-7 (Oriental Instruments Ltd., Tokyo). The surviving fraction was calculated by dividing the colony number of cells exposed to the drug by that of the control. The IC_{90} value of each drug was determined from the dose-response curves.

Flow cytometry Cells were seeded at about 2×10^5 cells/5 ml culture medium in 60-mm dishes. On the following day, the cells were continuously exposed to 1×10^{-5} m*M* CPT or 6×10^{-4} m*M* VP-16. After 24, 48 or 72 h, cell aliquots were incubated with 10 m*M* BrdUrd for 30 min at 37°C. A single-cell suspension was prepared by trypsinization, then the cells were fixed with 70% ethanol at 4°C for 30 min. The fixed cells were hydrolyzed in 3 *N* HCl at room temperature for 20 min, centrifuged and resuspended in 1 ml of 0.1 *M* $Na_2B_4O_7$, followed by two washes in PBS. The cells were then incubated at room temperature for 30 min in PBS containing 0.5% Tween 20, 0.5% bovine serum albumin, and mouse monoclonal anti-BrdUrd (Becton Dickinson Immunocytometry Systems, San Jose, CA; diluted 1:100), following which they were washed twice in PBS. They were then further incubated in PBS containing 0.5% Tween 20, 0.5% bovine serum albumin, and fluorescein-conjugated goat antimouse IgG (Cappel, Organon Teknika Corp., West Chester, PA; diluted 1:100) for 30 min at room temperature, followed by two washes in PBS. Finally, the cells were counterstained with 50 μg/ml of propidium iodide (Sigma Chemical Co.) in PBS, and studied by FCM after 30 min.

About 1×10^6 dual-stained cells were analyzed by Ortho Cytron flow cytometry (Ortho Diagnostic Systems Co., Tokyo). The laser was adjusted to emit approximately 500 mW at 488 nm. Green fluorescence was measured through a 514.5 nm bandpass filter and taken as a measure of the incorporated BrdUrd. Red fluorescence was measured through a 600 nm long-pass filter and was taken as a measure of the total DNA. The accumulated data were arranged into a bivariate 64×64 channel plot to show the cellular distribution of DNA and BrdUrd in each group.

RESULTS

Kinetic analysis of etoposide and camptothecin We analyzed CPT and VP-16 to determine kinetically whether or not the mode of cell-killing differs between these representative inhibitors of DNA topoisomerases I and II. WiDr cells were exposed to several concentrations of CPT or VP-16 for various periods, and surviving fractions were determined by a colony assay. The concentration-survival curves after various lenghts of exposure are shown in Fig. 1. The concentration-survival relationships of these drugs presented a sharp contrast. The cell-killing ability of VP-16 was dependent on both time and concentration; the concentration-survival curves constantly shifted to the left as the exposure period increased. On the other hand, the effect of CPT seemed to be dependent on time more than concentration. Potent cell-killing was observed at very low concentrations after continuous exposure for more than 16 h. According to our kinetic analysis, the IC_{90}s and the corresponding exposure times were plotted on log-log graphs (Fig. 2). As a reflection of the above concentration-survival relationships, VP-16 had an almost linear relationship with a slope of −1. The shape of this curve was similar to those of mitomycin C, cisplatin, nimustine, and some other alkylating agents, and is characteristic of cell cycle phase-nonspecific agents.[10, 11] Our previous kinetic analysis also

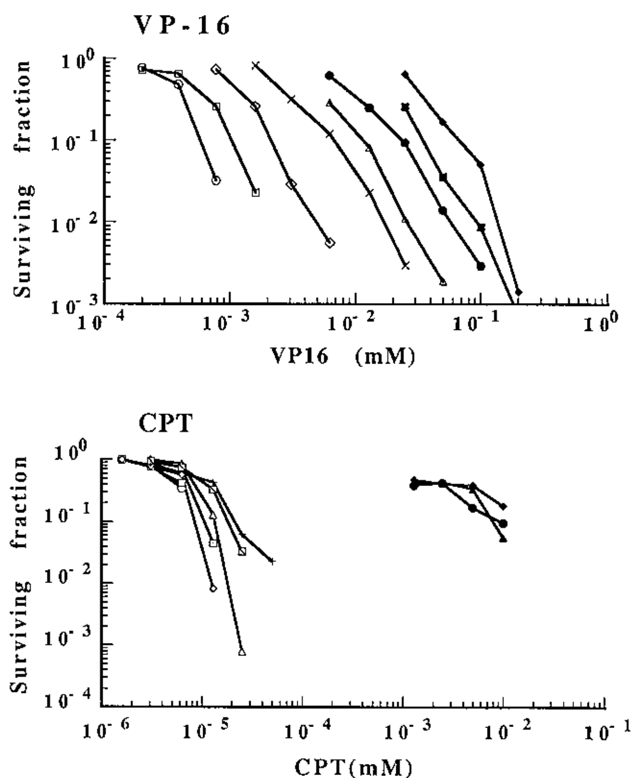

Fig. 1. Concentration-survival curves for various lengths of exposure to VP-16 and CPT. WiDr cells were exposed to varying concentrations of VP-16 or CPT for various periods, and the surviving fractions were evaluated by a colony assay. One of three sets of similar results for each drug is presented. Exposure times: 0.25 (◆), 0.5 (■), 1 (●), 3 (△), 6 (×), 18 (◇), 24 (□) and 72 (○) h for VP-16; 1 (◆), 4 (▲), 8 (●), 16 (×), 24 (⧅), 48 (△), 72 (⊡), 96 (◇), 144 (□), 216 (○) h for CPT.

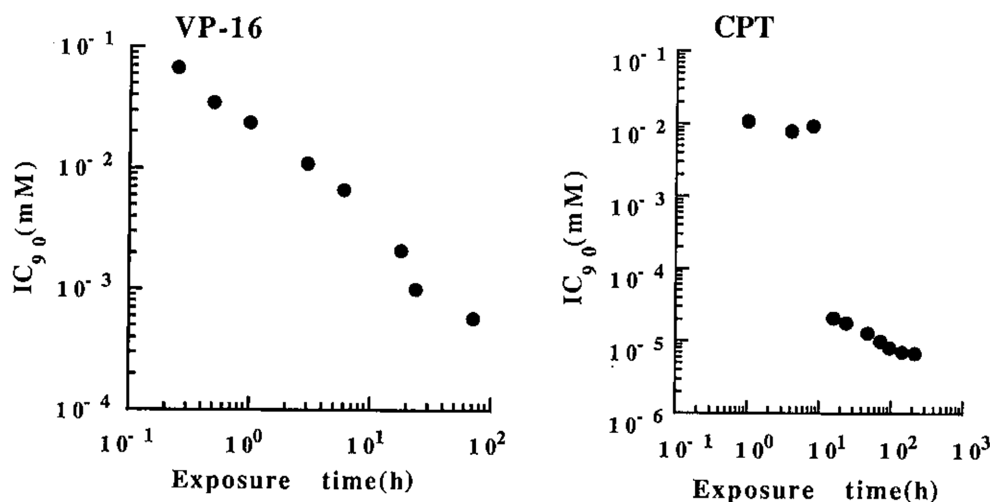

Fig. 2. Log-log relationship between the IC_{90} value and exposure time for VP-16 and CPT. The IC_{90} values obtained from the concentration-survival curves for VP-16 and CPT were plotted against exposure times, each on a log scale.

indicated that the cell-killing action of this class of agents is dependent on the product of concentration and time (C×T), or the AUC. In contrast to VP-16, the plot for CPT presented a curve with a very steep slope, which is similar to that of cytosine arabinoside.[11] This curve was regarded as being characteristic of cell cycle phase-specific agents, indicating that long-term exposure is indispensable for efficient cell-killing.

Kinetic analysis of other DNA topoisomerase I and II inhibitors To ascertain whether the difference in cell cycle phase-specificity of cell-killing effects between CPT and VP-16 is common to other inhibitors of DNA topoisomerases I and II, we studied several agents in a similar manner. Log-log plots of 3 inhibitors of DNA topoisomerase II, VM-26, m-AMSA and UCT4-A, and 2 inhibitors of DNA topoisomerase I, KT-6528 and UCE6, are shown in Figs. 3 and 4, respectively.

As shown in Fig. 3, the plots for VM-26, a very close analog of VP-16, and m-AMSA, a typical DNA topoisomerase II inhibitor with DNA-intercalating activity, were linear with a slope of about -1. On the other hand, the curve for UCT4-A, a novel non-intercalative terpenoid, which induces a heat-stable topoisomerase II-DNA cleavable complex,[15] had a plot with a slope more gentle than -1. According to our kinetic analysis, this profile indicates that UCT4-A is a cell cycle phase-nonspecific agent which was partially degraded *in vitro*. Thus the DNA topoisomerase II inhibitors studied act as cell cycle phase-nonspecific agents.

Two novel DNA topoisomerase I inhibitors were also studied. One was KT6528, one of the synthetic derivatives of the indolocarbazole antibiotic K252a[13] and the other was UCE6, an antibiotic with a naphthacene quinone structure.[14] Both compounds induce a potent topoisomerase I-mediated DNA cleavage comparable to that of CPT. As shown in Fig. 4, log-log plots for these topoisomerase inhibitors presented steep slopes similar to that of CPT, indicating that these 3 inhibitors of DNA topoisomerase I induce cell-killing in a cell cycle phase-specific manner.

Flow cytometry Changes in the cell cycle phase distribution by VP-16 and CPT during exposure to drugs were compared. The concentrations of VP-16 and CPT used for the continuous exposure were 6×10^{-4} and 1×10^{-5} m*M*, respectively, which are quite cytotoxic under these conditions. With VP-16, there was a prominent accumulation in G_2-M phase, as shown in Fig. 5. This change is characteristic of cell cycle phase-nonspecific agents such as alkylating agents, DNA intercalators, etc. In contrast, when cells were exposed continuously to CPT, they accumulated remarkably in late S-phase, suggesting S-phase specific action.

DISCUSSION

Previous biochemical studies of individual agents have indicated that inhibitors of DNA topoisomerases I and II kill cells by different mechanisms after forming cleavable complexes. However, the precise mode of action remains unknown, particularly that of topoisomerase II inhibitors. Here, we directly compared the cell-killing actions of DNA topoisomerase I and II inhibitors using a kinetic analysis which distinguishes between cell cycle phase-specific and -nonspecific agents. We showed that the

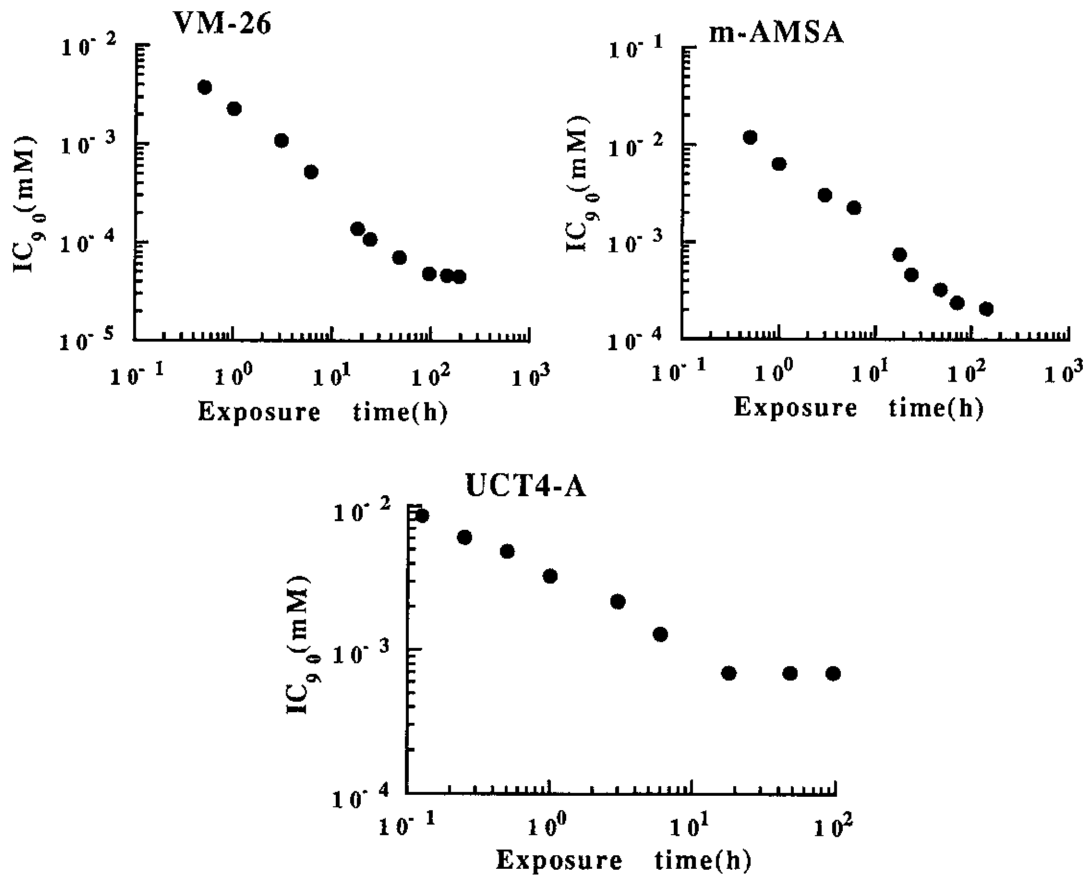

Fig. 3. Log-log relationship between the IC_{90} value and exposure time for DNA topoisomerase II inhibitors, VM-26, m-AMSA and UCT4-A. The IC_{90} values obtained from the concentration-survival curves for each agent were plotted against exposure times, each on a log scale.

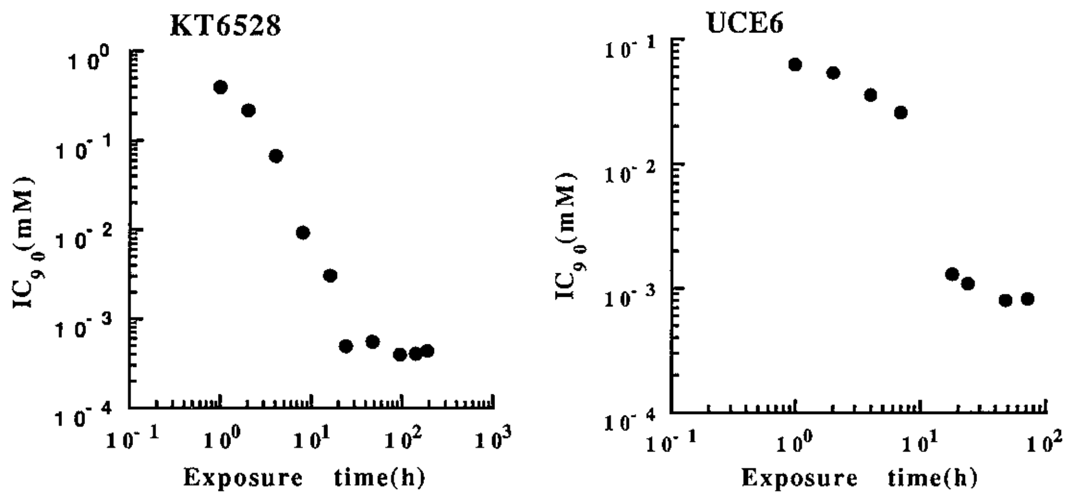

Fig. 4. Log-log relationship between the IC_{90} value and exposure time for DNA topoisomerase I inhibitors, KT-6528 and UCE6. The IC_{90} values obtained from the concentration-survival curves for each agent were plotted against exposure times, each on a log scale.

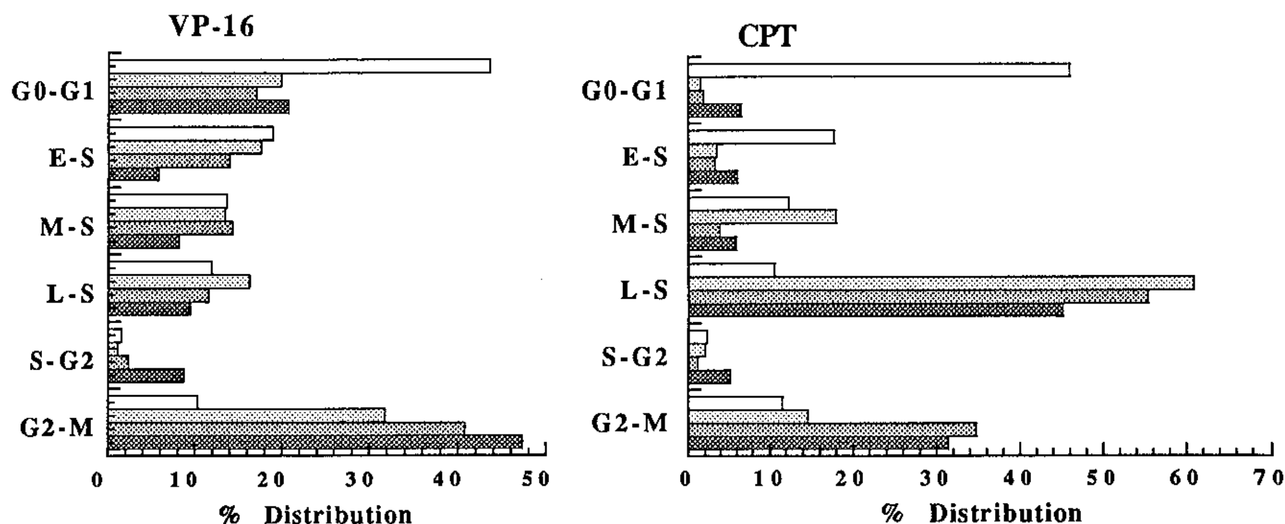

Fig. 5. Changes in the cell distribution at various cell cycle phases during exposure to VP-16 and CPT. WiDr cells were treated with 6×10^{-4} m*M* VP-16 or 1×10^{-5} m*M* CPT continuously up to 72 h, and the cell distribution in G_0-G_1, early S (E-S), middle S (M-S), late S (L-S), S-G_2, and G_2-M phases was measured in terms of the bivariate DNA/BrdUrd distribution at 0 (□), 24 (▤), 48 (▧) and 72 (▩) h.

DNA topoisomerase I inhibitors, CPT, KT6528 and UCE6 kill cells in a cell cycle phase-specific manner, whereas the DNA topoisomerase II inhibitors, VP-16, VM-26, m-AMSA and UCT4-A do so in a cell cycle phase-nonspecific manner. These results suggested that the different characteristics could be generalized as being those of topoisomerase I and II inhibitors which form cleavable complexes, since several drugs belonging to the same category exhibited common behavior in our analysis.

The present results also indicated that the cell-killing effects of DNA topoisomerase II inhibitors are dependent on the AUC. Therefore, in principle, their antitumor effects *in vivo* are not significantly influenced by treatment schedule. On the other hand, those of DNA topoisomerase I inhibitors do not depend on the AUC but upon the length of exposure rather than the concentration. Accordingly, a long exposure to DNA topoisomerase I inhibitors is required for efficient cell killing. However, frequent administration or long infusion is not always necessary for *in vivo* treatment by DNA topoisomerase I inhibitors if they have a high binding capacity for serum proteins. It is noteworthy that clinically available CPT analogs have potent protein-binding ability.

Although DNA topoisomerase I and II possess some different features regarding structure, ATP requirement, major type of DNA strand breaks induced, etc., they have a common critical role in basic cellular functions which include DNA replication, recombination and transcription. The two classes of inhibitors form cleavable complexes with DNA and the respective enzyme, stopping both DNA and the enzymes from functioning. From this perspective, the different modes of cell-killing action between DNA topoisomerase I and II inhibitors are difficult to understand.

One possible explanation for the different cell-killing modes is that formation of cleavable complexes by DNA topoisomerase I and II inhibitors occurs in S phase alone, and in all phases of the cell cycle, respectively. This hypothesis requires the S phase-specific appearance or increase of the intracellular level of DNA topoisomerase I and a constant level of DNA topoisomerase II during the cell cycle. However, this is not the case for DNA topoisomerase I or II[16, 17]; the former did not show any significant fluctuations in content or stability across the cell cycle, and the latter exhibited marked degradation as cells progress from mitosis to G_1 phase although keeping a constant level in the remaining phases. Thus, the notion of S phase-specific formation of cleavable complexes by DNA topoisomerase I inhibitors may not reasonably explain their cell-killing actions.

Another possible explanation for the present results is related to the biochemical mechanisms of the cytotoxicity of the inhibitors. Hsiang *et al.* have proposed that a collision between moving replicatoin forks and cleavable complexes formed by CPT causes irreversible arrest of the replication fork, thus inducing cell kill.[5] When CPT was combined with aphidicolin, an inhibitor of DNA polymerase-α and -δ, CPT cytotoxicity was prevented without affecting the formation of cleavable complexes.[5, 6, 8] This suggests that active DNA synthesis

is required for CPT cytotoxicity. Based upon this mechanism, the results of our kinetic analysis of DNA topoisomerase I inhibitors can be explained.

Aphidicolin provided only partial protection against VP-16- and m-AMSA-induced cytotoxicity.[8] Instead, inhibitors of DNA transcription and protein synthesis prevented their cytotoxicity. Co-exposure to a transcription inhibitor, 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole or cordycepin, protected cells from the cytotoxicity induced by m-AMSA, but not that by CPT, without reducing cleavable complex formation.[6] On the other hand, cycloheximide, a protein synthesis inhibitor, also abrogated VP-16- and m-AMSA-induced cytotoxicity.[7, 9] Furthermore, Bertrand *et al.* have reported that calcium-dependent cellular processes, probably relating to signal transduction, are required for VP-16 cytotoxicity.[18] Thus, the precise mechanisms of the cell-killing action of DNA topoisomerase II inhibitors following cleavable complex formation remain to be elucidated, but seem more closely connected with RNA and protein synthesis than with DNA synthesis, as compared with the action of CPT. This situation can account for the cell cycle phase-nonspecific actions of these drugs that we found in this study.

Tanabe *et al.* have reported that bis(2,6-dioxopiperazine) derivatives such as ICRF-154 and -193 inhibit DNA topoisomerase II without forming cleavable complexes.[19] This new type of DNA topoisomerase II inhibitor was most effective against cells in the G_2-M phase, and produced typical multinucleated cells.[20] Independently of their studies, we found that ICRF-154 shows cell cycle phase-specific action using our kinetic analysis.[21] Accordingly, the conclusion in the present study is limited to DNA topoisomerase inhibitors of which the cell-killing actions are mediated by the cleavable complex.

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